THE VARIATION IN THE UNIT OF THE ŒSTRUS-PRODUCING HORMONE.

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The introduction of the vaginal smear test on ovariectomised rats or mice for extracts containing the œstrus-producing hormone has led to its widespread use at the present time for the determination of the relative potency of different preparations. Doisy, Ralls, Allen and Johnston(1) have defined as a "Rat unit" the "quantity of material necessary to induce œstrus as judged by the smear method in an ovariectomised, sexually mature rat weighing 140 ± 20 grm." It is evident that the value of their unit is dependent on the assumption that the smallest dose able to produce œstrus in one rat will not seriously differ from that required in another.

Certain observations made by one of us in an attempt to determine the mouse unit of different preparations, suggested that for ovariectomised mice at least this assumption was incorrect. Three results, which were simultaneously obtained, appear in Table I. The results with all

Table I.

Extract No. 1			Extract No. 2			Extract No. 3		
Mouse	Dose	Result	Mouse	Dose	Result	Mouse	Dose	Result
1	40 mgm.	Œstrus	7	40 mgm.	Œstrus	13	40 mgm.	Œstrus
2	20 „	„	8	20 „	Nil	14	20 „	Nil
3	15 „	„	9	15 „	Œstrus	15	15 „	„
4	10 „	Nil	10	10 „	Nil	16	10 „	„
5	7·5 „	Œstrus	11	7·5 „	Œstrus	17	10 „	„
6	5 „	Nil	12	5·0 „	„	18	5 „	Œstrus

three extracts were irregular, but consideration of those obtained with Nos. 2 and 3 showed that the method applied in this way offered no hope of arriving by the use of a few animals at a trustworthy determination of the minimum effective dose. It was evidently necessary to carry out an investigation of the variation between different animals.

Trevan(2) has shown that tests such as the frog test for digitalis and

the mouse test for insulin must, if grave errors are to be avoided, be carried out in such a way that the variation of different animals is not neglected but is made the basis of the test. He has shown that since frogs vary in their response to digitalis by 300 p.c., an effort to determine the minimal lethal dose by giving a series of diminishing doses cannot be expected to give a correct result. It is necessary first to determine for the species of frog used a standard curve relating the percentage mortality of frogs to the dosage. Having obtained this standard curve, it is then possible to assay an unknown sample by injecting *e.g.* 40 frogs with one dose. The potency of the sample is then determined by noting the abscissa on the standard curve corresponding to the percentage mortality which occurs.

In this paper we have applied the principles laid down by Trevan to the investigation of the rat and mouse unit, and for this purpose have examined one sample of ovarian extract on 90 rats and 70 mice.

Methods.

1. *Selection of animals.* One hundred young does were obtained from an average healthy stock of rats of mixed black and white strain. They were 7–8 weeks old and weighed 60–100 grm. They had been fed from weaning on a mixture of crushed oats 25 p.c., barley meal 25 p.c., middlings 35 p.c., and a proprietary food called vitamealo 15 p.c., with about three drops of cod liver oil per rat twice a week. After being brought to the laboratory the same diet was continued, with the daily addition of about 1 cub. in. of bread which had been soaked in milk and squeezed out. The addition of about six drops of a crude cod liver oil to the mixed diet twice a week was continued throughout the experiment. Growth proceeded at an average rate to maturity. Of the hundred animals the complete series of observations was made on 90.

2. *Examination of smears.* The vaginal smears were examined by the method described by Long and Evans(3), which one of us (K.H.C.) had the privilege to learn in Prof. Evans' laboratories in California. The smear is rubbed from the spatula on to a drop of tap water or saline on a microscope slide and the constituents are identified through the low power objective at once. No drying or staining is necessary. The smears from 18 to 20 rats can be made in separate drops of water on one large slide, and one hundred rats can be examined by two workers in half an hour.

3. *The removal of the ovaries.* The operations were carried out under deep anæsthesia with ether and with aseptic precautions. The rats

recovered completely within an hour, and their wounds healed by first intention in every instance. It was interesting to note that in several rats œstrus appeared within two days, but never more than two days, after ovariectomy.

4. *The œstrus-producing hormone used.* The preparation of œstrin (as Parkes and Bellerby (4) suggest the hormone should be called) was very kindly placed at our disposal by Mr F. H. Carr, C.B.E., F.I.C., of the British Drug Houses. It was a bulk of about 10 grm. of extract which was kept by Mr Carr *in vacuo* over sulphuric acid at 0° C. A day or two before each injection was made, a portion of the bulk was weighed in a tared glass tube, and olive oil was added to a mark, so that the weight of extract per c.c. of oil was accurately known. For injection a known volume of oil was emulsified in a mortar with 1 p.c. anhydrous sodium carbonate in sufficient amount to allow the desired weight of extract to be injected in a volume of 0·4 c.c. for every rat, and of 0·2 c.c. for every mouse. The dose was administered under the skin as a single injection. Local disturbances at the site of injection in the form of induration or necrosis were not observed, and there was no indication of incomplete absorption of any dose.

5. *The determination of œstrus.* The change in the smear which was considered sufficient to indicate that œstrus had resulted from an injection of the extract was either (*a*) the absence of leucocytes, and their replacement by either nucleated or a mixture of nucleated and cornified cells on one day, followed on the next day by a mixture of leucocytes and cornified cells; or (*b*) the appearance of nothing but cornified cells on one day. These changes, when they occurred, were complete within 72 hours of the injection.

Experimental results on rats.

A small portion of the extract used was first tested by injection into five ovariectomised rats other than the rats already described. Of two of these which received 2·5 mgm. of the extract, one passed through the œstrous cycle. It seemed likely that an injection of 5·0 mgm. would lead to the occurrence of œstrus in the majority of rats, and this dose was prepared for the first injection. All the rats were injected with this one dose at a time six days after the last and fifteen days after the first of the operations for ovariectomy had been performed.

After an interval of 10 days, another dose was chosen and given to every rat. At successive intervals of 14 days other doses were given in the same way. The results appear in Table II.

TABLE II.

Date	Dose per rat	No. of rats	No. in which œstrus was observed
9th Dec.	5·0 mgm.	90	32
19th Dec.	2·5 „	90	7
3rd Jan.	7·5 „	90	31
17th Jan.	12·5 „	90	55
31st Jan.	17·5 „	90	76
14th Feb.	10·0 „	90	40

It was a matter of considerable surprise to find that the injection of 5·0 mgm. was followed by œstrus in only 32 rats. The result of injecting 2·5 mgm. was consistent with this, but the injection of 7·5 mgm. was followed by œstrus in one less rat than the injection of 5 mgm. The impression was given that a loss in sensitiveness had taken place. The very slow increase in the number of rats giving the response as successively larger injections were made further suggested that there was a progressive loss of sensitiveness, but the result of the injection of 10 mgm. on 14th Feb. showed that so far as the period following 19th Dec. was concerned there had been no loss of this nature. For when a curve (see Fig. 1) was drawn through the points obtained by plotting the dose

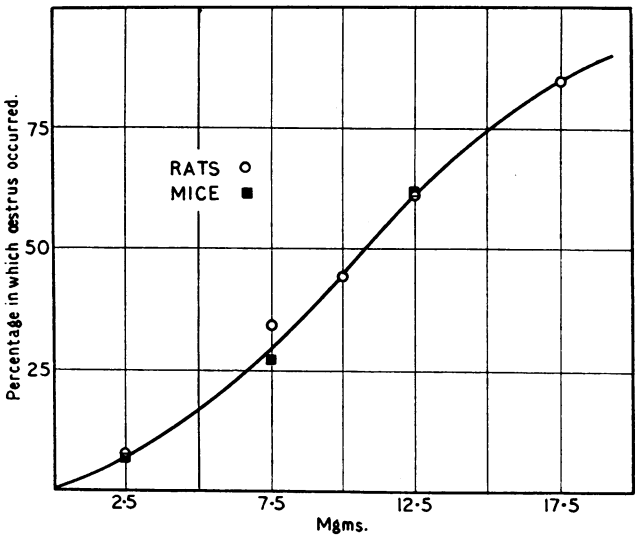

Fig. 1.

against the percentage of rats in which œstrus occurred, it was found that all points except that of 9th Dec. lay on a well-defined curve. Had there been any progressive decrease in sensitiveness the injection of

10 mgm. on 14th Feb. would have given a result which when plotted would have lain below the curve. It has already been mentioned that the first injection followed ovariectomy by only six days in some of the rats, whereas the remaining injections were separated by 14 day intervals. Analysis of the figures showed that the disagreement between the result of the first injection and that of the later ones was due to this difference, and that for the purpose of establishing the variation in rats, the result of the first injection must be set aside as being complicated by an additional factor.

The coincidence of the result of the injection of 14th Feb. with the predetermined curve, showed in addition that there had been no loss of activity of the extract used during its stay *in vacuo* at 0° C.

The results showed that not only was it untrue that the minimal effective dose of œstrin was approximately the same for all rats, but that the divergence between different rats was unusually wide. Seven times the amount of œstrin sufficient to produce œstrus in 7 p.c. of animals, was still insufficient to produce œstrus in more than 84 p.c. of animals.

Experimental results on mice.

Several observers have preferred to use the mouse, and it seemed of importance to make parallel observations on this animal to determine with accuracy (*a*) whether mice showed the same variation as rats, (*b*) the relation of the mouse unit to the rat unit. The experiments were performed on 70 mice, all obtained from one dealer at one time, in exactly the same way as those on the rats. The operations for ovariectomy were completed on 20th Dec. and the first injection was not made until 14 days afterwards. As before, 14 days intervened between successive injections. The oestrin was prepared as for the rats, the material injected being a portion of the sample used for injection into the rats on the same day.

The results appear in Table III.

Table III.

Date	Dose per mouse	No. of mice	No. in which œstrus occurred
3rd Jan.	2·5 mgm.	70	5
17th Jan.	7·5 „	70	19
31st Jan.	12·5 „	70	43

It will be seen from Fig. 1, that when these observations are expressed as a percentage of mice and plotted against the dose, the points lie on the same curve as that given by the rats.

Relation of variation to body weight and other factors.

The doses of œstrin injected were not calculated according to the body weight of each animal either for rats or mice. It seemed possible, at first sight, that the variation observed between rats might to some extent be due to this, and that on analysis it might be found that it was lighter animals only which responded to the lower doses. Each rat had been weighed once a fortnight throughout the experiment, and examination showed that there was no correlation between body weight and the dose necessary to produce œstrus. Thus the injection of 12·5 mgm. of the extract on 17th Jan. produced œstrus in 55, and failed to do so in 35 rats. The average weight of the 55 rats was 150 grm.; the average weight of the 35 was slightly, but not significantly less. The weights in the two groups were distributed fairly evenly about the same weight, 150 grm.

The group of rats in which œstrus occurred had on the whole grown rather more in the previous six weeks, but the difference was again not significant.

Vaginal smears of all the rats had been taken daily for three weeks before ovariectomy, and during this period some rats were seen to pass through one or more cycles, while in others no cycle was observed. It was not found after ovariectomy that the former group was the more, or that the latter was the less sensitive to œstrin.

Regeneration.

A factor which might have complicated the results was regeneration of ovarian tissue after ovariectomy. Smears were taken daily in rats from the time of operation for 34 days. For the next six weeks they were examined only on the day before injection and the next five days. From this point they were examined daily for 25 days. Regeneration was observed in two rats only, and these are not included in the 90 animals on which the results were obtained. During the injection of the mice, smears were again only examined on six days; 14 days after the last injection, a daily examination was made, again for 25 days; there was evidence of regeneration in five mice only, which is a much smaller number than had been expected from the results of Davenport(5).

Intraperitoneal injection.

Evans and Burr(6) have published a short paper giving results on a few animals from which they conclude that the dose necessary to

produce œstrus is larger if it be injected into the peritoneal cavity than if injected under the skin. As they were apparently unaware of the great variation in rats, it was possible that their result was merely a demonstration of this variation, and not of the difference between the two modes of administration. The hypodermic injection of 10 mgm. of the extract into the rats on 14th Feb. produced œstrus in 44 p.c. On 28th Feb. this amount was administered by intraperitoneal injection, and œstrus was produced in 48 p.c. The difference between the two results is not significant, and the results show that it is immaterial whether the injection be made under the skin or into the peritoneal cavity.

Injection in three doses.

Evans and Burr (*loc. cit.*) give illustrations confirming the previous suggestion of Doisy, Ralls, Allen, and Johnston (*loc. cit.*) that a smaller amount of extract is sufficient to produce œstrus if it be injected in three separate doses at intervals of four hours, than if it is injected in one single dose.

Fourteen days after the intraperitoneal injection just described, 50 rats received by hypodermic injection a total of 10 mgm. given in three doses at four-hour intervals. Œstrus followed in 19, that is in 38 p.c. This figure is lower than that obtained by a single injection of 10 mgm. into 90 rats, which was 44 p.c., but here again the difference is not significant. It is evident that the single is at least as efficient as the triple injection.

The variation in the response of single rats.

In view of the variation in the sensitiveness of different rats, it was most important to discover whether each animal behaved in a reasonably uniform way. The evidence on this point is on the whole depressing. In the first place the behaviour of the rats may be considered in giving the results which appear in Table II. If the result of the first injection be excluded for the reasons already stated, then in the course of the other injections 30 out of the 90 animals behaved irregularly, in the sense that doses higher than the smallest which produced œstrus did not invariably produce œstrus. From the 60 rats, which were regular in this sense, should be deducted 14 in which œstrus never occurred at all, so that out of 76 animals which may be used as evidence, three-fifths were regular and two-fifths were irregular.

Further information is given by the three injections each of 10 mgm. The second of these was an intraperitoneal injection, and for the third

three injections of 3·3 mgm. were given at intervals of four hours. Since the results were fairly uniform (œstrus occurred in 44, 48 and 38 p.c. respectively) it may be assumed that the method of injection made no difference, and that the same results would have followed a single hypodermic injection in each instance. If this be granted, then of the 50 rats which were used in all three observations, only 19 gave the same response each time. For the first two observations 90 rats were used, and in these 51 animals behaved in the same way both times. It is clear that the sensitiveness of any one animal undergoes very considerable variations.

The method of assaying an extract.

The rat unit may be redefined as that amount of extract which produces œstrus in 50 p.c. of ovariectomised rats. In order to examine an unknown sample, 20 rats are chosen from the stock, 10 of which do not usually respond to an injection of one unit, and 10 of which usually do respond to this amount. To each of the 20 rats the same dose is given, and the number in which œstrus occurs is observed. Suppose this be x, so that the percentage is $5x$. The abscissa corresponding to the ordinate

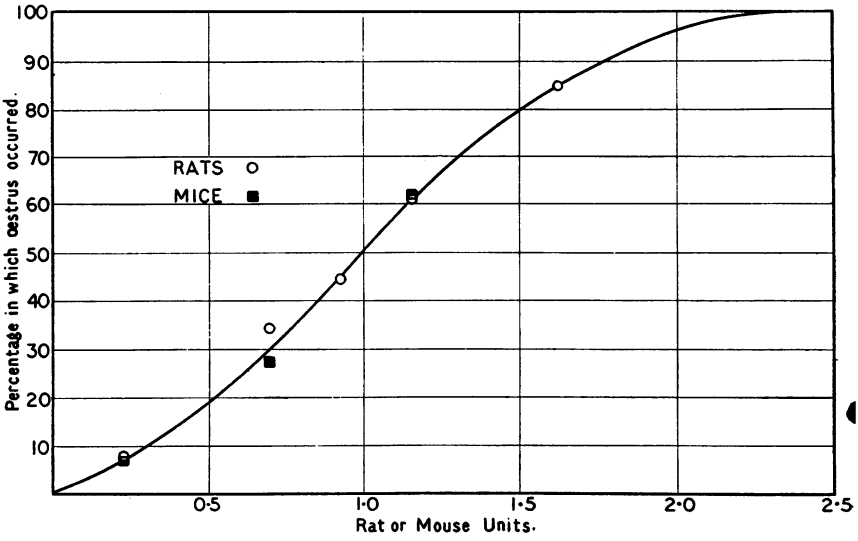

Fig. 2. Standard curve showing relation of units injected to effect produced.

$5x$ is then noted on the curve shown in Fig. 2. This abscissa gives the number of units present in the dose injected.

The rats used for this assay are injected every fourteenth day, and at

no other time. There is at present no trustworthy evidence to show the effect on sensitiveness of discontinuing the injections for any period.

Discussion.

It has been found that the variation in the rat unit as defined by Doisy, Ralls, Allen and Johnston (*loc. cit.*) is more than 700 p.c. and appears from the curve shown in Fig. 2 to be about 1000 p.c. That this great variation is not due to accidental selection of unusual animals appears from the fact that precisely the same variation has been found in the mouse unit. From the investigation has emerged the very striking observation that the average rat unit is the same as the average mouse unit. This fact is significant for the understanding of the action of œstrin. That the same amount of œstrin which just suffices to induce a response in a mouse should also be enough to bring about the same change in an animal 7·5 times as big, differentiates its action sharply from that of insulin, the dose of which is closely proportional to the body weight. It is clear that the factor which determines the size of the minimum effective dose is not the necessity for a certain concentration of the hormone in the body tissues or fluids.

The variation in different animals greatly complicates the assay. Provided however that different workers determine the average rat or mouse unit in the manner described, there should be a certain degree of resemblance between the results of different workers, inasmuch as rats in different countries are not likely to be more dissimilar than rats and mice in Great Britain. It should be recognised, however, that even if the average unit be determined, unless this is done on approximately 100 animals, there may still be serious discrepancies between different workers, and these will remain until a standard sample of œstrin is available for distribution by a central international authority. When this is prepared it may be prophesied that by a general consensus of opinion the unit will be redefined as the amount of activity present in a certain weight of the standard preparation. Having determined in 20 animals the number which respond to a given dose of the standard, the strength of an unknown sample will then be determined in terms of the standard by injecting the 20 animals with a dose of that sample.

The variation which may occur in determining the average unit at the present time can be calculated from the formula for the standard deviation $\sqrt{\frac{p.q}{N}}$ where p is the percentage of animals in which œstrus has occurred, q is the percentage in which it has not occurred, and N is

the number of animals. If 25 animals are used, and œstrus occurs in 50 p.c., the standard deviation will be 10 p.c. This means that if the experiment be repeated several times, on different rats, then twice out of three times the percentage of animals in which œstrus occurs will be not less than 40 or more than 60. But once in twenty-two times the percentage will differ by more than twice the standard deviation, and be less than 30 or greater than 70. Thus if only 25 animals are used, once in twenty-two experiments a dose which is actually one average unit will appear as less than 0·7 units or as more than 1·3 units, and the error in the assay will be more than 30 p.c.

SUMMARY.

1. An investigation of 90 rats and 70 mice has shown that the variation in the rat unit or the mouse unit may be as great as 1000 p.c.

2. The unit is redefined as the dose required to produce œstrus in 50 p.c. of ovariectomised animals.

3. The relation of the average rat unit to the average mouse unit is one.

4. The average unit is the same whether the injection be made as a single subcutaneous injection, as a single intraperitoneal injection or as a triple subcutaneous injection of which the separate injections are made at intervals of four hours.

5. Single animals, injected once a fortnight with the same dose, vary considerably in their response.

6. The method used in this laboratory for determining the unit is described.

We wish to express our thanks to our assistant, Mr H. W. Ling.

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